# ABSTRACT


Title of Thesis: EFFECTS OF POST-HARVEST MANAGEMENT PRACTICES ON THE DEGRADATION OF *BACILLUS THURINGIENSIS* PROTEINS IN GENETICALLY MODIFIED CORN RESIDUE

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Most studies addressing the ecological effects of Bt crops have focused on non-target effects during the crop growing cycle. Less information is available on the fate of expressed toxins in crop residue after harvest in a no-till production system. This research investigated the effects of four post-harvest management practices on the degradation rates of Cry proteins expressed in SmartStax field corn. Cry protein degradation expressed as growth inhibition of *Ostrinia nubilalis* larvae after harvest was measured by a feeding bioassay, and enzyme-linked immunosorbent assays were used to detect the presence of Cry proteins. Cry proteins retained significant levels of biological activity at crop senescence and were still present in corn residue for more than 20 weeks after harvest. Despite inconsistencies in treatment effects, the study demonstrated that postharvest practices that increase soil-residue contact increase protein degradation, thereby reducing the period of exposure for non-target organisms.

# EFFECTS OF POST-HARVEST MANAGEMENT PRACTICES ON THE DEGRADATION OF *BACILLUS THURINGIENSIS* PROTEINS IN GENETICALLY MODIFIED CORN RESIDUE

by

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## *Introduction*

### Genetically engineered crops

Significant developments in agricultural biotechnology over the past three decades have resulted in the rapid adoption of genetically engineered (GE) crops in the United States and worldwide. The most common transgenic traits convey either insect pest resistance via plant-incorporated protectants or herbicide tolerance. Insect resistant transgenic crops contain single or pyramided crystalline (Cry) and vegetative insecticidal (VIP) proteins expressed by genes isolated from the soil bacterium *Bacillus thuringiensis* (Bt). The first genetically engineered corn (herein noted as Bt corn) was introduced commercially in 1996 and expressed the lepidopteran-active Cry1Ab toxin. The Bt transgenic technology is now a major pest management tool in agricultural crop production. Bt corn plantings in 1997 represented roughly 8% of the total corn acreage in the U.S., and by 2017 more than 90% of all corn acreage was genetically engineered (NASS 2017). Similarly, Bt cotton acreage in the US has increased from 15% in 1997 to 80% in 2017 (NASS 2017). The highly effective control of many lepidopteran and coleopteran pests provided by Bt transgenic technology has reduced pest populations and insecticide usage, conserved biocontrol agents, and

provided economic benefits to farmers (Shelton et al. 2002, Cattaneo et al. 2006, Hutchison et al. 2010, Kathage and Qaim 2012, Perry et al. 2016, Dively et al. 2018).

However, the widespread production of Bt crops has raised many questions about potential environmental risks, with particular emphasis on the ecological effects of the expressed proteins on non-target organisms. There are several published reviews and meta-analyses that summarize the current knowledge regarding the effects of Bt crops on non-target organisms (Wolfenbarger et al. 2008, Marvier et al. 2007, Yu et al. 2011, Comas et al. 2014, Romeis et al. 2014). The majority of non-target studies have focused on changes in individual taxa abundance or community structure during the Bt crop's growth cycle. In general, these reviews have found no adverse effects of Bt crops on the abundance of non-target organisms compared to the negative effects of insecticide-treated controls. The current literature contributes substantially to our understanding of potential non-target effects of Bt crops. However, there is less information available on the fate of expressed toxins and their exposure to non-target organisms in soil or crop residue after harvest. To date, studies investigating the effects of Cry proteins from Bt crops on post-harvest invertebrate communities have found no consistent or lasting adverse effects on a range of soil organisms including woodlice, collembolans, mites, earthworms, nematodes and protozoa (Saxena and Stotzky 2001, Icoz and Stotzky 2008, Bai et al. 2012, Clark and Coates 2006, Honemann et al. 2008). The potential exposure and impact of crop residue containing Bt proteins entering aquatic systems have

also been addressed in studies over the past decade (Rosi-Marshall et al. 2007, Parrott 2008, Beachy et al. 2008, Swan et al. 2009, Chambers et al. 2010). Bt proteins have been found in aquatic systems (Tank et al. 2010) and some aquatic invertebrates have been shown to be affected (Jensen et al. 2010, Axelsson et al. 2011). Although studies to date show no convincing evidence of adverse effects from the expressed Bt protein in crop residue on non-target organisms, further research is needed to fully understand the movement and fate of Bt proteins for the regulatory risk assessment process (Rose 2007).

A major development in transgenic corn technology over the past decade is the addition of stacked and pyramided Bt corn hybrids which suppress a broader spectrum of pest populations (Carriere et al. 2016). Notwithstanding, the persistence of multiple toxins present in senescing corn may result in a longer period of exposure to nontarget organisms. In no-till production systems typical of the mid-Atlantic region, corn residue that remains after harvest is equivalent to ~27 kg per bushel of harvested grain or 10 metric tons per hectare if corn yields exceed 375 bushels per hectare (Hickman and Schoenberger 1989). This residue represents the primary source of Bt proteins entering agricultural soils (Zwahlen et al. 2007). However, this residue can move out of crop fields via wind and heavy rainfall, subsequently becoming a source of toxin exposure to non-target organisms in surrounding woodlands and streams.

**Corn residue decomposition**

The amount of corn residue remaining on the soil surface or incorporated into the soil is reduced over time by microbial decomposers. Studies that

examined the decomposition rates of Bt and non-Bt post-harvest corn litter have reported conflicting results. Several studies determined that Bt corn residue contains increased lignin and therefore decomposes at a slower rate than their respective non-Bt isolines (Saxena and Stotzky 2001, Fang et al. 2007, Castaldini et al. 2005, Flores et al. 2005, Poerschmann et al. 2005). Lignin compounds are more resistant to microbial decomposition compared to other plant compounds (Coyne 1999), leading some researchers to test the hypothesis that higher lignin content will result in a reduced decomposition rate. Another explanation as to why Bt corn decomposes slower is that stalks are not damaged by the European corn borer (*Ostrinia nubilalis*), and consequently are less infected with stalk rots which are known to increase decomposition of harvested corn residue. Flores et al. (2005) used gross metabolic activity of the soil as an indicator of decomposition by measuring $CO_2$ evolution in soils amended with Bt and non-Bt corn tissue. Soils amended with Bt residues were found to produce significantly lower amounts of $CO_2$ than those amended with non-Bt tissues. Similarly, Dinel et al. (2003) also measured $CO_2$ evolution in soils under Bt and non-Bt crops and found that the cumulative $CO_2$ flux from Bt soils was 30.5% lower than soils under non-Bt crops. Taken together, any reduction in the rate of residue decomposition could increase the period of exposure to soil and surface dwelling organisms.

Other studies have found lower or no differences in lignin content in Bt corn leaves compared to their near isolines (Escher et al. 2000, Jung and Sheaffer 2004, Mungai et al. 2005). Moreover, many studies have reported no differences

in the decomposition rate of Bt and non-Bt corn residue (Daudu et al. 2009, Hopkins and Gregorich 2003, Tarkalson et al. 2008, Zwahlen et al. 2007, Honemann et al. 2008, Lehman et al. 2008, Zurbrugg et al. 2010). A three-year study by Xue et al. 2011 also observed no significant differences among Bt, non-Bt, and non-Bt + insecticide on initial tissue lignin concentrations, decomposition rate, or bacterial decomposer communities. They concluded that any differences observed were likely due to environmental factors and not differences among corn hybrids. The combined evidence to date indicates that the presence of Bt proteins does not influence the decomposition dynamics of the corn residue.

Studies have shown significant effects of environmental factors such as temperature, precipitation and residue-soil contact on decomposition of post-harvest corn litter. In a litter bag study investigating decomposition under various tillage systems, Burgess et al. (2002) found that buried litterbags containing post-harvest corn tissue lost mass significantly faster than those that remained on the soil surface, indicating an accelerated effect of soil contact on the rate of tissue decomposition. Another study investigating winter decomposition of transgenic cotton residue in conventional-till and no-till systems found that Bt and non-Bt cotton residues decomposed significantly faster in conventionally tilled compared with no-till fields (Lachnicht et al. 2004). Residues remaining on the soil surface also experience greater fluctuations in temperature and moisture content, thus reducing the amount of time where conditions are optimal for tissue decomposition (Summerell and Burgess 1989, Parr and Papendick 1978).

**Bt protein persistence and degradation**

Knowledge of the persistence of insecticidal Cry proteins in corn residue after harvest is essential for assessing the ecological risk of transgenic technology on nontarget organisms. Root exudation and incorporation of senesced plant litter after harvest represent two major sources of carbon for agricultural soils (Icoz and Stotzky 2008). They also represent the major mechanisms by which active Bt proteins are introduced into the soil rhizosphere (Saxena and Stotzky 2000). Protein degradation rates in the soil are influenced by a variety of environmental factors including temperature, soil type, the particular protein being examined (Sanvido et al. 2007) and the extent of tissue incorporation into the soil. Like any plant protein, Cry proteins generally degrade faster when exposed to soil organisms. A study of the effects of environmental conditions on Cry protein degradation determined that Cry1Ab proteins degrade faster at higher temperatures, but are not affected by soil water content or pH (Feng et al. 2011). Temperature was also found to significantly affect Cry1Ac protein degradation in transgenic rice residues (Li et al 2007). This study found that protein concentrations decreased rapidly in the first month after harvest, then gradually after that. Degradation was found to increase again, however, when temperatures increased the following spring. Li et al. (2007) also found that Cry1Ac proteins degraded more slowly in water than in soil, suggesting that slower protein degradation may occur in litter that has entered aquatic systems. When examining Cry1Ac protein degradation under different soil water content settings, Zhang et al. (2015) found that high soil water content resulted in reduced protein

degradation, with 100% water holding capacity resulting in significantly lower amounts of Cry1Ac degradation than those under 50% and 70% water holding capacity.

Studies designed to determine how long Cry proteins persist in the soil have produced conflicting results (Icoz and Stotzky 2008). Under certain environmental conditions, active Cry proteins have been detected in the soil rhizosphere of field grown Bt corn plants up to several months after the crop has been harvested. Saxena and Stotzky (2001**)** determined that Bt toxins from plant root exudates and degrading biomass persists in the soil for up to 350 days. Another study detected Bt toxins from transgenic cotton in the soil up to 140 days after harvest when the experiment was terminated (Palm et al. 1996). Alternatively, Head et al. (2002) found that the amount of Cry1Ac that accumulated in the soil from three to six years of repeated transgenic cotton planting and subsequent incorporation was below the limit of detection for bioassay and enzyme-linked immunosorbent assay (ELISA) tests. Similarly, Dubelman et al. (2005) found no evidence of persistence or accumulation of Cry1Ab proteins in soils from fields planted with Bt corn hybrids for at least three consecutive growing seasons. To date, few studies on the persistence of Cry proteins in soil have identified the soil properties that determine persistence (Dubelman et al. 2005; Shan et al. 2008). Hung et al. (2016) tested the persistence of Cry1Ac in three different soil types under a range of temperatures and determined that temperature was of much greater importance than soil type, with higher temperatures promoting faster degradation. Stotzky (2005) determined that

Cry proteins produced by transgenic plants bound tightly and rapidly to clays, humic acids extracted from the soil, and on complexes of clay and humic acids. Once bound, the Cry proteins retain biological activity and are less susceptible to microbial degradation. The potential for long term Cry protein persistence in agricultural soils has raised concern about their potential impact on non-target organisms. Yet, little information is available on the fate of Cry proteins in no-till corn systems under different postharvest conditions.

**Justification and objectives**

The widespread and repeated use of Bt corn in no-till rotational systems requires continued efforts to assess the non-target effects of toxin expression in post-harvest plant residue. A risk assessment of post-harvest exposure involves the determination of decomposition rates of the transgenic tissue and qualification of the biological activity of Bt proteins over time in order to assess how long non-target organisms may be exposed after harvest. Additionally, the amount, physical structure and soil contact of the surface residue may be affected by post-harvest management practices (i.e., mowing, tillage, cover cropping) that are commonly used in no-till corn systems. To my knowledge, there are no studies addressing how these post-harvest practices impact the decomposition rates of corn residue or the biological persistence of Bt proteins. It is possible that certain post-harvest management practices can shorten the exposure period of active Bt proteins, thus reducing the potential risk to non-target organisms.

To better understand factors affecting the post-harvest fate of Bt proteins, I conducted research to determine how long biologically active Bt proteins persist

in senesced corn residues when subjected to four post-harvest management practices. The most common practices used in the fall following harvest are some form of tillage, mowing, cover cropping; or leaving the corn residue undisturbed on the soil surface following combine harvest. Leaving the plant residue undisturbed after harvesting during the fall reduces erosion by keeping the maximum amount of residue on the soil surface. However, excess corn residue without soil contact is slower to decompose, can insulate the soil and reduce warming and drying in the spring. This may prevent early planting of the subsequent crop (Soane et al. 2012). Too much corn residue on the surface can also result in reduced or non-uniform emergence of the following crop. Mowing with a flail-type mower can enhance more uniform residue coverage over the surface, more direct contact of the residue with the soil, and help reduce plant stand issues the following year. Conventional tillage using a chisel or moldboard plow incorporates large amounts of litter into the soil, speeding up decomposition of plant material. Agronomy extension publications generally state that 5-10% and 60-80% of the original amounts of corn residue remains on the surface after tillage operations using a moldboard and chisel plow, respectively (Hickman and Schoenberger 1989, Al-Kasi et al. 2002). Planting a cover crop in late summer or fall following crop harvest is a common practice in the mid-Atlantic region to prevent soil erosion, reduce nutrient runoff and protect water quality. When the cover crop is no-till planted, the action of the drill incorporates roughly 10% of the residue into the soil while cutting it into smaller pieces that have more direct soil contact. Once plants emerge, cover cropping also creates a

favorable, moist microclimate under the canopy for decomposers to increase the rate of residue decomposition. Surveys conducted in 2016 and 2017 (CTIC 2017) determined that 88% of farmers reporting across the US use cover crops, primarily planted after harvest, and 82% of these farmers practiced no till or conservation tillage systems. I hypothesized that corn residue will decompose faster and the Cry proteins present in corn residue will lose biological activity at a higher rate if subjected to postharvest management practices that increase the residue-soil contact. Additionally, the degradation of Cry proteins depends largely on microbial activity, which decreases at colder temperatures (Zwahlen et al. 2003). Therefore, I also predicted less degradation to occur during winter months.

For each post-harvest management practice, I used enzyme-linked immunosorbent assays (ELISA) to determine the presence of Bt toxin in corn residue samples collected over time and also performed laboratory feeding bioassays with a sensitive insect species (European corn borer) on the same samples to assess biological activity. ELISA assays are widely used to monitor the presence of Bt proteins in plant tissue because they are relatively inexpensive, convenient to use, and produce quick results (Albright et al. 2016). Non-target studies have used results of ELISA assays as evidence of bioactive Bt proteins present in environmental samples and reported an exposure risk to sensitive non-target organisms. However, assays can produce false-positives indicating the presence of proteins that have been structurally altered and not biologically active. Studies by Marchetti et al.(2007) and Gruber et al. (2011) showed that the

bioactivity of Bt proteins decreased more rapidly than what was indicated by ELISA results, indicating that the ELISA assay was detecting degraded, non-bioactive forms of the proteins. Thus, many studies have not performed any form of biological validation to determine if Bt proteins detected in environmental samples by ELISA were biologically active. For this reason, I compared results of both assay methods, and predicted that the ELISA will detect the presence of Bt proteins in corn residue tissue for a longer period after harvest than the feeding assay.

*Materials & Methods*

**Field Site**

The experiment was conducted in the same field from 2014-2015 and 2016-2017 during the fall and winter seasons at the Central Maryland Research and Education Center in Beltsville, MD. The study was also conducted during the 2015-2016 season. However, most experimental plots were destroyed by a fire when post-harvest treatments were being applied. The field site (39.012420°,-76.825712°) is located in the Atlantic Coastal Plain eco-region. Total precipitation was approximately 612 mm during sampling months in 2014/2015 and 506 mm in 2016/2017. Soils at the field site are Elsinboro series fine-loamy, mixed, semiactive, mesic Typic Hapludults. The field had historically been under a no-till corn-soybean rotation system.

**Test corn hybrids**

In both years, Dekalb® SmartStax (DKC62-08) was used as the genetically engineered hybrid and its closely related non-Bt isoline (DKC62-05)

as a control. This SmartStax hybrid contains three Bt proteins [Cry3Bb1 (event 88017), Cry34Ab1 (event DAS-59122-7) and Cry35Ab1 (event DAS 59122-7)] that are active against corn rootworms (*Diabrotica sp.*); three proteins [Cry1A.105 (event MON 89034), Cry2Ab2 (event MON 89034), and Cry1F (event TCI507)] active against a complex of lepidopteran pests; and two traits providing tolerance to glufosinate and glyphosate herbicides. All seeds were treated with a standard fungicide combination and 0.25 mg a.i. per kernel of clothianidin (Poncho 250, Bayer CropScience) to control soil insects and diseases. Plots were planted May 20 (2014) and April 26 (2016) into a killed cover crop with a six-row John Deere no-till planter set at a seeding rate to achieve 65,000 corn plants per ha. In 2014, the cover crop consisted of 25 kg. cereal rye, 4 kg. crimson clover and 1 kg. tillage radish, while a different seed mixture of 31 kg. cereal rye, 4 kg. crimson clover and 1 kg. tillage radish was used in 2016. In both years, the cover crop was killed by applying Gly Star Plus at 2,000 $cm^3$ (glyphosate), Sharpen at 59 $cm^3$, and Sim-Trol at 0.5 kg (simazine) 2 weeks before corn planting. All plots were managed under a fertility program consisting of 16-8-8 (N-P-K) starter fertilizer applied at 228 kg per ha at planting, followed by a side-dress application of 148 kg per ha of 30% nitrogen solution dribbled between the rows three weeks later. Paraquat (Gramoxome Extra, Syngenta Crop Protection) at 0.82 kg per ha was used as a burn down herbicide, and atrazine plus S-metolachlor (Bicep II Magnum, Syngenta Crop Protection) at 2.18 kg per ha was applied after planting to control weeds. Corn was machine harvested with a John Deere s660 combine in late September when plants senesced and kernel

moisture was less than 20%. The combine was equipped with a straw spreader that evenly distributed the corn refuge over a swath that was roughly equal to the width of the combine head.

**Experimental Design**

The experimental layout was a modified split-plot design with hybrid type (SmartStax or non-Bt isoline) as whole plot factors and post-harvest management practices following harvest as the subplot factors. Four subplots, each measuring 9.14 meter wide (12 rows) by 15.24 meters long, were arranged in alternating whole plots of SmartStax and non-Bt isoline hybrids (Fig. 1). Subplot treatments included: 1) a single pass of a Brillion chisel plow that breaks up and stirs soil roughly 38 cm beneath the soil surface, which partially incorporated the corn residue (chisel plow); a single pass of a flail mower using staggered T-shaped cutting blades to completely shred the standing corn stalks and surface residue into a uniform mulch (flail mow); 3) flail mowing followed by a cover crop planting of a rye, crimson clover and radish mix, seeded with a Great Plains 1510 No-till drill in narrow rows 19 cm apart (cover crop); and 4) undisturbed crop residue that remained after harvest (undisturbed).

Each treatment was replicated three times. All plots in 2014 were directly adjacent to each other, and subplots in 2016 were separated by a 9.14 meter wide non-crop buffer within each whole plot to allow for equipment movement (Fig 2). Within one week after grain harvest, four post-harvest management practices were randomly assigned and applied to subplots within each whole plot treatment. In 2014, the amount of plant residue on the soil surface was measured by

randomly placing a 0.42 $m^2$ area circular frame and collecting all plant material within the frame. Samples were taken from each subplot, except for the undisturbed treatment, which was assumed to represent similar amounts of surface residue recorded in flail mowed subplots. Each residue sample was brought back to the laboratory and weighed to record the wet weight.

The purpose of the chisel plow treatment was to assess the fate of Bt proteins in corn residue buried in the soil, typical of the soil incorporation resulting from a moldboard plow. However, it was not operationally feasible to sample soil-incorporated tissue in the chisel plow plots; thus, buried litterbags were used as an alternative method (Dadu et al 2009). Eight mesh litterbags were filled with approximately 100 g of corn residue collected from each subplot within a few days prior to the chisel plow treatment. In 2014, only leaf tissue was collected, whereas a more representative, proportional mixture of leaf and stalk tissue was collected in 2016. After chisel plowing, the eight bags were placed 0.6 m apart and buried to a depth of 15 to 20 cm in the center of each subplot. For the remaining treatments, approximately 100 g of leaf tissue was collected within each subplot on each sampling date in 2014, except for the flail-mowed treatment, where different plant parts could not be differentiated; thus, all available surface residue was collected. Similarly, a proportional mixture of leaf and stalk tissue was collected from the surface in all treatments in 2016.

In both years, samples of corn residue were collected from Bt and non-Bt plots after harvest to measure the protein activity before postharvest treatments were applied. Additional leaf samples were taken in 2014 at anthesis and when

kernel moisture reach 30% prior to harvest to measure the reduction in protein activity during corn senescence. After the postharvest treatments were applied, samples of surface residue and the content of litterbags were collected every two weeks for the first eight weeks, and then every four weeks thereafter until mid-April. After each collection, samples of leaf tissue or postharvest residue were brought back to the laboratory, cut into small pieces, and homogenized. A subsample was then removed, frozen at -80°C. and then processed in a LABCONCO freeze drier model 195 at 2 x $10^{-3}$ mBar vacuum pressure to lyophilize the tissue to prevent denaturing of the Bt proteins. The lyophilized Bt and non-Bt tissue was then ground to a fine powder in a commercial blender (IKA Works, Inc, Wilmington, DE) and stored at -80 C until used in bioassays.

**Enzyme-Linked Immunosorbent Assay (ELISA) Testing**

Samples of Bt residue tissue from the replicate plots of each treatment and sampling date were tested individually using an enzyme-linked immunosorbent assay [Quad Trait ImmunoStrip (ELISA), Agdia Inc., Elkhart, IN], which detected the presence or absence of Bt-Cry1F, Bt-Cry2A, Bt-Cry34Ab1 and Bt-Cry3Bb1 proteins. For each sample, 0.02 grams of ground, lyophilized tissue were individually placed into small centrifuge tubes along with 1 milliliter of 10% buffer solution. Each tissue mixture was vortexed for 15 seconds and then centrifuged for 2 minutes at 8,000 reps per minute. The solution of each sample was transferred into individual vials, and the kit-provided ELISA strips inserted for 10 minutes. At test completion, a darker control line indicated a valid test, while light but clearly visible test lines were interpreted and recorded as a positive

test for the presence of each Cry protein. All ELISA strips were preserved for documentation.

**Feeding bioassay testing**

A standard feeding bioassay described in Jensen et al. (2010) was performed to measure the growth inhibition of European corn borer (*O. nubilalis*) as a sensitive indicator for the presence of biologically-active Cry proteins remaining in the tissue samples. *O. nubilalis* eggs were obtained from a commercial insectary (Benzon Research, Carlisle, PA), placed in plastic deli cups containing a meridic diet for *O. nubilalis* (Southland Products Inc, Lake Village, AR), and incubated in a growth chamber at 25ºC until early 2$^{nd}$ instar larvae were available for bioassays.

Three replicates of all treatment by hybrid type samples of each sampling date (n=24) were assayed on the same day. For each bioassay, 1200 ml of the meridic diet (adjusted with more water to offset the added tissue) was prepared and cooled in a water bath at 55ºF. Pre-weighed quantities of 600 mg of each lyophilized tissue sample were prepared and placed in labeled plastic weight trays. For each sample, 25 ml of molten diet was drawn into a 60 cc plastic syringe with a 4 ml diameter tip opening. The tip was then capped and the plunger removed in order to add the tissue powder into the syringe body. With the plunger re-inserted, the syringe was shaken back and forth for 10 sec and then held firmly for 15 seconds on the rubber platform of a vortex mixer to thoroughly homogenize the tissue-diet mixture. The incorporation dilution resulted in a concentration of 24 mg of lyophilized tissue per ml of diet. Approximately 1.5 ml

of the tissue-diet mixture of each sample was dispensed into each of 16 wells of a 128-well bioassay tray (C-D International). After the diet mixture cooled and solidified, one early second instar was transferred to each well using a camel-hair brush. Each 16-well section (consisting of 4 rows and 4 columns) of the bioassay tray was sealed with a perforated adhesive cover, and then trays were held in a growth chamber at 25ºC, L:D 14:10, and 40±60% RH. After seven days, all live larvae within each row of four wells were recovered and weighed together, and the weight gain per larva was calculated by dividing the pooled weight by the number of larvae in each row. The weight gain was then averaged across all four rows for each sample. For each bioassay, three replicate groups of 10 early second instars were weighed together to calculate the average initial weight per larvae, which was used to adjust the weight gains after 7 days.

**Statistical analyses**

For each bioassay sample, absolute and relative values of weight gain of larvae exposed to Bt tissue were directly compared to that of unexposed larvae in the same replicate block. A three-way mixed model ANOVA (SAS Institute 1997) tested for main and interaction effects of hybrid type, post-harvest treatment, and sampling week on larval weights. The larval weight data was then expressed as percent growth inhibition resulting from exposure to Cry proteins by calculated the difference in mean larval weight gain of cohorts feeding on diets incorporated with Bt and non-Bt tissue. The difference was divided by the larval weight gain of cohorts feeding on non-Bt tissue diet and then multiplied by 100. A two-way mixed model ANOVA tested for main and interaction effects of post-

harvest treatment and sampling week on percent growth inhibition. Before each analysis, data were tested for normality and homogenous variance using the Shapiro-Wilk *W* test, Spearman's rank correlation, and by examining residual plots. Data transformations and grouping of variance were performed as necessary. For each analysis, sampling week was modeled as a repeated measure, and replicate blocks were treated as a random factor. Significant effects among means were separated by using Tukey's adjustment for pair-wise comparisons ($P \leq 0.05$).

The ELISA data, expressed for each sample as 1= positive detection or 0= negative detection, were analyzed by ANOVA using SAS PROC GLIMMIX to test for differences in the proportion of positive detections among postharvest treatments over sampling weeks. A separate analysis was conducted for each protein (Cry1F, Cry2A, Cry34Ab1 and Cry3Bb1), assuming a binary distribution of the response variable. In each analysis, the r-side modeling approach and MMPL estimation method were used, postharvest treatment, sampling week and their interaction were treated as fixed effects, and a RANDOM statement modeled the repeated measures of binary outcomes. Means were estimated using the LSMEANS statement and tested for differences at $\alpha = 0.05$ by the pdiff option.

*Results*

**Plant Residue on Soil Surface**

Based on observations in both years, the crushing and shredding of stalks at the combine head removed the ears and a portion of the stalks and leaves, leaving roughly 25 to 30 cm of the basal portion of stalks and roots intact and

attached. The material that passed through the combine consisted of corncobs, husks, intact pieces of cornstalks, and smaller chaffy pieces. The spinning disks of the chaff spreader distributed the discharged material more uniformly behind the combine. Although not quantitatively measured, visual assessments estimated that 20-30% of the plant residue, particularly standing stalks and lower leaves, did not have direct contact with the soil surface. The flail mower chopped and flattened the standing plant residue, increasing more direct contact with the soil but without incorporation. The total amount of corn residue on the soil surface in the flail mowed treatment plots in 2014 (also representative of the undisturbed plots) averaged 11.09 ± 0.96 metric tons per hectare. This residue amount was significantly higher than the other post-harvest treatments ($F_{(2, 1)}$=18, $P$<0.0001). The chisel plow treatment incorporated 60.7% of the crop residue into the soil, with an average 4.36 ± 0.73 metric tons of residue per hectare remaining on the soil surface. The planting of the cover crop incorporated 11.67 % of the residue into the soil, with an average 9.79 ± 0.52 metric tons of residue per hectare remaining above ground. The action of the drill buried a portion of the corn residue into the seed furrows, resulting in slightly less surface residue than the undisturbed and flail mowed plots.

**Bioassay Results – Year 1**

Results for bioassays conducted using green tissue collected when corn plants began silking at growth stage R1, and again roughly 20 days prior to harvest at growth stage R6 are summarized in Table 1. Percent inhibition at growth stage R1 was nearly 99% when compared to larvae reared on similar stage

non-Bt tissue. Percent growth inhibition at the R6 stage was nearly 98%, indicating little degradation of the Cry proteins occurred in the leaf tissue prior to harvest.

Data for the 7-day feeding bioassays for all post-harvest sample weeks were summarized as larval weight gains for both hybrid types and also as relative growth inhibition resulting from exposure to Cry proteins for each sampling week and treatment combination. Figure 3A displays the three-way interaction means for weight gain of larvae fed a diet incorporated with Bt and non-Bt corn residue. This interaction was not significant, indicating that postharvest treatment differences between hybrid types were relatively consistent at each sampling week. However, the interaction between hybrid and sampling week pooled across treatments was highly significant ($F_{(8,141)}$=6.64, $P$<0.001), showing a steady increase in weight gain and progressively smaller differences between hybrid types across sampling weeks. The weight gain of larvae feeding on diet incorporated with Bt and non-Bt corn residue averaged 0.08 ±0.08 and 60.92±2.28 mg at week 0 and increased to 60.92±5.52 and 87.98±3.23 mg at week 25, respectively. Pooled over sampling weeks, Figure 3B shows an overall postharvest treatment effect on the weight gain of larvae exposed to Bt corn residue but no effect on larvae exposed non-Bt residue ($F_{(3, 78.4)}$=3.15, $P$=0.030). Similarly, the weight gain differences among postharvest treatments over sampling weeks for larvae exposed only to Bt corn residue were nearly significant for the interaction effect ($F_{(24,45.9)}$=1.73, $P$=0.055). Generally, larvae exposed to Bt residue from the chisel plow and undisturbed treatment plots gained less body

weight compared to larvae exposed to the other treatments at most sampling dates (Fig. 4A). Bioassay results for larvae exposed only to Bt corn residue shows significant main effects for sampling week ($F_{(8, 59.3)}$=38.17, $P$<0.0001) and the postharvest treatments ($F_{(3,36.5)}$=8.38, $P$<0.001). As shown by the trend in Figure 4A, weight gain was lowest in all treatments at week 0 and increased significantly throughout the duration of the study. Furthermore, overall weight gain throughout the entire 25-week sampling period was numerically lower for larvae exposed to Bt corn residue collected from chisel plow and undisturbed treatment plots (Fig. 4B). These larvae weighed 68% less than the larvae exposed to non-Bt residue from the same treatments.

Figure 5 summarizes the Year 1 bioassay results expressed as the percent growth inhibition resulting from exposure to Cry proteins in corn residue collected from each sampling week and treatment combination. Larval growth was inhibited more than 96% by the Bt toxin expression in the senesced corn residue prior to the application of the postharvest treatments at week 0. Bioassay results of residue samples collected after the postharvest treatments showed a pattern in treatment differences that was relatively consistent from week 4 through week 22, as indicated by a nonsignificant treatment by week interaction effect (Fig. 5A). Generally, corn residue from the chisel plow and undisturbed plots resulted in the highest growth inhibition. As expected, there was a significant week effect ($F_{(8, 70)}$=9.15, $P$<0.001), showing a steady decline in overall percent inhibition from 97.16% at week 0 to 28.56% at week 25. Pooled over weeks, the postharvest treatments had a significant main effect on larval

growth inhibition ($F_{(3, 70)}$=3.73, $P$=0.015) as shown in Figure 5B. Overall inhibition was greatest, but not statistically different, in the chisel plow (70.92% ± 5.59) and undisturbed treatment (69.37% ± 4.49). Weight gain of larvae fed diet incorporated with Bt corn residue from the cover crop and flail mowed treatments experienced 58.47% ± 5.92 and 54.36% ± 5.27 growth inhibition, respectively. Only corn residue subjected to flail mowing caused a significantly lower amount of growth inhibition compared to the chisel plow and undisturbed treatments.

**ELISA Results– Year 1**

Qualitative ELISA testing was performed on all residue samples from the Bt hybrid plots to detect the presence of Cry3Bb1, Cry34Ab1, Cry2A and Cry1F proteins. Raw data results of individual replicate tests for each treatment and sampling week combination are depicted in Figure 6. The data for each protein type, recorded for each sample as 1= positive detection or 0= negative detection, were averaged and plotted in Figure 7 as the probability of positive detection to show trends among postharvest treatments over sampling weeks. All residue samples collected at week 0 prior to the application of postharvest treatments tested positive for each Cry protein. Conversely, all samples tested at week 22 were negative, indicating that each Cry protein was no longer present. It was not possible to test for treatment by week interaction effects when analyzing the detection data separately for each protein, because the GLIMMIX analysis could not converge. However, there was clear evidence that the rate of positive detections varied considerably among the different Cry proteins. For comparison, the overall probability of detection for each protein type

was averaged over weeks and treatments, excluding weeks 0 and 22. The Cry3Bb1 protein consistently showed the lowest detection rates for all postharvest treatments, with an overall probability of detection averaging 12.5% ± 0.05. In contrast, the probability of positive detections for Cry34Ab1, Cry2A and Cry1F proteins averaged 90.3% ± 0.08, 59.7% ± 0.16 and 80.6% ± 0.11% , respectively, with most of the negative detections occurring after week 6. Pooled over protein types and weeks, probabilities of positive detections for samples collected in the cover crop, chisel plow, flail mow and undisturbed treatments averaged 50.0% ± 0.07, 66.7% ± 0.06, 54.2% ± 0.07 and 72.2% ± 0.04, respectively. Pooled over treatments, there were significant sampling week effects for all proteins, as evident by the decline in positive detections particularly after week 6.

Significant main effects for postharvest treatments depended on the protein type. For Cry3Bb1, overall probabilities of detections ranged from 5.6 to 16.7% and were not significantly different among treatments. Similarly, the postharvest treatments had no significant effect on the presence of Cry34Ab1, which was detected in 77.8% to 100% of the samples. However, the persistence of the Cry2A ($F_{(3,56)}$= 231.1, $P$<0.001) and Cry1F ($F_{(3,12)}$= 7.31, $P$=0.005) proteins were significantly affected by the postharvest treatments. Overall positive detection probabilities for Cry2A averaged 44.4% for samples collected from both cover crop and flail mow plots, whereas 72.2% and 66.7% of the cover crop and flail mow samples tested positive for Cry1F, respectively. In comparison, detection levels were significantly higher for Cry2A and Cry1F in the chisel plow

(66.7 % and 88.9 %, respectively) and the chisel plow and undisturbed plots (83.3% and 94.4 %, respectively).

**Bioassay Results – Year 2**

Data for the 7-day feeding bioassays performed during the 2016/2017 seasons were also summarized as larval weight gains for both hybrid types and as relative growth inhibition resulting from exposure to Cry proteins for each sampling week and treatment combination. Figure 8A displays the three-way interaction means for weight gain of larvae fed a diet incorporated with Bt and non-Bt corn residue. Unfortunately, samples from the chisel plow plots were lost and not analyzed for weeks 2, 4, and 8. Due to the unbalanced design, the three-way analysis involved only data from weeks 8 through 24. As in year 1, this three-way interaction was not significant, indicating that postharvest treatment differences between hybrid types were relatively consistent at each sampling week. The interaction between hybrid and sampling week pooled across treatments for sampling weeks 8 through 24, however, was highly significant ($F_{(4, 69.1)} = 6.6$, $P<0.001$), showing a steady increase in weight gain and progressively smaller differences between hybrid types across sampling weeks 8 through 24. A full analysis of data prior to week 8 was not possible due to the chisel plow missing samples; however, trends in weight gain for the remaining three postharvest treatments were very similar, showing a steady increase in weight gain until week 4 or 6, and then followed by abrupt declines at week 8. Overall, weight gain of larvae feeding on Bt or non-Bt residue incorporated diet averaged 5.92 ± 3.80 and 62.51 ± 5.99 mg at week 0 but later treatment effect converged to

levels averaging 21.44 ± 1.54 and 29.97 ± 1.38 mg at week 24, respectively. Pooled over sampling weeks 8 through 24, Figure 8B shows an overall postharvest treatment by hybrid effect on larval weight gain, with larvae exposed to Bt residue weighing significantly less then larvae exposed to non-Bt residue, except for the chisel plow treatment ($F_{(3, 39.9)}$=19.79, $P$<0.001). This interaction had no effect on larvae exposed to non-Bt residue for all treatments. An interaction effect was also significant for the weight gain differences among postharvest treatments over sampling weeks 8 through 24 for larvae exposed only to Bt corn residue ($F_{(12, 65)}$=7.16, $P$<0.001) (Fig. 9A). Overall weight gain results for larvae exposed only to Bt corn residue from weeks 8 through 24 show significant main effects for sampling week ($F_{(4, 66.4)}$=39.73, $P$<0.001) and postharvest treatment ($F_{(3, 77.3)}$=65.96, $P$<0.001). As shown in Figure 9A, weight gain was lowest in all treatments at week 0 and increased significantly during sample weeks 2, 4 and 6 for the cover crop, flail mow and undisturbed treatments. Interestingly, weight gains for these treatments were much lower in week 8 compared to previous sample weeks, but larvae exposed to Bt tissue significantly increased throughout the remaining sampling weeks, except for the chisel plow treatment. Moreover, overall weight gain averaged over the 8-24 week period was statistically higher for larvae exposed to Bt corn residue collected from the chisel plow plots compared to the other 3 treatments (Fig. 9B).

Figure 10 summarizes the percent growth inhibition results of larvae feeding on diet incorporated with Bt corn residue subjected to different postharvest treatments. Prior to the application of postharvest treatments at week

0, Bt toxin expression in the senesced corn tissue resulted in about 90% inhibition of normal larval growth (Fig. 10A). Generally, bioassay results of residue samples collected during week 8 through week 24 postharvest showed a relatively consistent pattern in treatment differences, indicated by a nonsignificant treatment by week interaction effect. Although it was not possible to compare all treatments due to the missing chisel plow samples, bioassays of corn residue from this treatment consistently resulted in the least amount of larval growth inhibition after week 6, while percent inhibition generally the highest from residue samples collected from the flail mow and undisturbed plots. Pooled over all treatments, there was an overall steady decline in percent growth inhibition from 89.73% at week 0 to 19.46% at week 24. Based on data from weeks 8 through 24, the postharvest treatments had a significant main effect on larval growth inhibition ($F_{(3, 38)}$= 18.01, P <0.001) as shown in Figure 10B. Overall inhibition was lowest in the chisel plow treatment compared to all other treatments (9.76% ± 4.32). Larvae reared on diet incorporated with Bt corn residue from the undisturbed, flail mow and cover crop treatments experienced similar levels of inhibition, averaging 55.66% ± 5.41, 53.09% ± 5.56 and 48.72% ± 4.80 growth inhibition, respectively.

**ELISA Results – Year 2**

Qualitative ELISA testing was also performed on all residue samples from the Bt hybrid plots to detect the presence of Cry3Bb1, Cry24Ab1, Cry2A and Cry1F proteins. The raw data of individual replicate tests for each treatment and sampling week combination for Year 2 are given in Figure 11. Sample data for each protein type was recorded as 1= positive detection or 0 = negative detection.

Data was averaged and plotted in Figure 12 as the probability of positive detection to show trends among postharvest treatments over sampling weeks. Note that mean bars are missing for chisel plow samples before week 8. Of the other postharvest treatments, all residue samples collected at week 2 in the cover crop and flail mow treatments tested positive for each Cry protein, whereas all but one replicate of the undisturbed treatment samples tested positive for the Cry3Bb1 protein. Positive detections continued in all treatments through week 24 for at least one of the four proteins tested but declines in the probability of positive detection varied over weeks depending on the protein type and postharvest treatment. The GLIMMIX analysis again could not reach a modeling solution to test for treatment differences among protein types over sampling weeks; however, there were obvious trends showing different declining rates of positive detections over time among the different Cry proteins. The persistence of the Cry3Bb1 and Cry2A proteins declined at a faster rate in all postharvest treatment plots compared to the other two proteins. Cry3Bb1 and Cry2A showed the lowest detection rates for all postharvest treatments, with an overall probability of detection averaging 23.3% ± 0.15 and 43.3% ± 0.27, respectively. Moreover, the presence of Cry34Ab1 and Cry1F proteins was generally detected over time in all treatment plots, except for the chisel plow samples, which showed positive detections only for Cry34Ab1. The overall probability of positive detections for the Cry34Ab1 and Cry1F proteins averaged 78.3% ± 0.13 and 68.3% ± 0.40, respectively. Pooled over protein types and weeks, the probabilities of positive detections for samples collected in the cover crop, chisel plow, flail mow and

undisturbed treatments averaged 60% ± 0.07, 15% ± 0.03, 63% ± 0.08 and 75% ± 0.07, respectively.

GLIMMIX analyses were able to statistically test for overall postharvest treatment effects on the probability of positive detections averaged over weeks 8 through 24. Significant main effects for postharvest treatment were significant for all proteins but differences varied among treatments. For Cry3Bb1, overall probabilities of detection ranged from 0 to 26.7% and were significantly lower in the chisel plow treatment ($F_{(3, 56)}$=367.9, $P$<0.001). Similarly, probability of detection for the Cry2A and Cry1F proteins ranged from 0 to 73.3% and 0 to 93.3%, respectively, with the average probability of positive detection also significantly lower in the chisel plow treatment samples (Cry2A: $F_{(3, 56)}$=452.2, $P$<0.001; Cry1F: $F_{(3, 12)}$=283.5, $P$<0.001). However, average detection probabilities for Cry34Ab1 were significantly higher for samples collected in the undisturbed plots (93.3%) compared to the cover crop (73.3%) and chisel plow (60%) plots ($F_{(3, 12)}$=14.1, $P$<0.001) but not statistically different from the flail mow treatment (86.7%).

*Discussion*

Overall results of the feeding bioassay and ELISA tests confirm that Cry proteins expressed in Bt SmartStax corn retained significant levels of biological activity at crop senescence. Over 97% growth inhibition of *O. nubilalis* larvae was observed when fed corn tissue collected immediately after harvest in 2014. ELISA tests also indicated 100% positive detections for all Cry proteins at this time. Similarly, larval growth was inhibited by over 89% in 2016 from the

presence of lepidopteran-active Cry proteins detected in corn tissue collected two weeks after harvest. Given that the concentration of lyophilized corn tissue in the bioassay diet was approximately 2.4%, this demonstrates the sensitivity of *O. nubilalis* larvae for measuring biological activity of lepidopteran active Cry proteins. Furthermore, expression levels in corn residue at harvest are 52-66% less for Cry2Ab2 and more than 98% less for Cry1F compared to peak levels of each protein during the R4 growth stage (EPA 2010a, EPA 2010b).

In both years, overall levels of Cry protein detection and larval growth inhibition declined with increasing weeks after harvest. Correspondingly, overall weight gains of larvae exposed to diet-incorporated Bt corn tissue steadily increased with increasing weeks after harvest. Averaged over the post-harvest treatments, percent growth inhibition was 48.1% ± 8.5 at week 22 of the first year and 31.5 ± 6.8 at week 20 of the second year. Taken together, this study provides strong evidence that biologically active proteins remained present in corn residue more than 20 weeks after harvest. These results are in disagreement with many studies addressing amounts and persistence of Cry proteins in the soil, submitted by registrants and reviewed for the current registrations of Bt corn events. For example, one study submitted by Monsanto used field-collected soils spiked with 500-fold excess of Cry2Ab2 protein and a corn earworm bioassay to quantify degradation. Results indicated that Cry2Ab2 protein concentration decreased by 50% in 1 to 6 days, and by 90% in 3 to 14 days in the three different soils (EPA2010b). Although this suggests that Cry proteins quickly degrade in artificially spiked soil, the degradation and loss of biological activity expressed in

decomposing corn residue may behave differently. Because crop residue typically stimulates microbial activity (Cheng and Coleman 1990, Griffiths et al. 1998, Jensen and Soerensen 1994), degradation rates of Cry proteins under field conditions is expected to be higher than those reported in studies using spiked protein in bulk soils. Sims and Holden (1996) found this to be true and reported that the 50% degradation time (DT50) of spiked Cry protein in bulk soil was about 5-fold higher than in Bt corn tissue added to the soil. Palm et al. (1996) also found more rapid degradation of Cry1Ab or Cry1Ac proteins when incorporated with cotton crop residues compared to when purified protein was used. Results of this study show that Cry proteins in corn residue with less soil contact (typical of no-till corn production) are more persistent and retain biological activity much longer than what has been reported in the literature. It should be noted that most soil fate studies in support of registration were designed to quantify Cry protein degradation when corn residue is incorporated into the soil, resulting from post-harvest tillage practices typical of corn production in the Corn Belt.

I hypothesized that the Cry proteins expressed in SmartStax corn would degrade and lose biological activity at a higher rate in post-harvest treatments resulting in greater soil-residue contact. It is generally known that Cry proteins degrade quicker if exposed to soil microbial activity. Thus, the buried litter bag tissue simulating the soil incorporated residue resulting from the chisel plow treatment was predicted to show the most rapid degradation. However, treatment effects were not consistent between years. In the 2014/2015 experiment, overall weight gains of *O. nubilalis* larvae exposed to Bt corn residue were lower, percent

growth inhibition was higher, and there were generally higher probabilities of Cry protein detection in the chisel plow and undisturbed treatments over the entire sampling period, compared to the cover crop and flail mow treatments. In 2016/2017, results of the bioassay and ELISA tests indicated a significantly higher rate of Cry protein degradation in the chisel plow treatment compared to the other postharvest treatments. Analysis of the litter bag residue showed the undetectable presence of Cry3Bb1, Cry2A and Cry1F proteins starting in week 8; overall weight gains of *O. nubilalis* larvae exposed to residue were significantly higher; and percent growth inhibition was significantly lower. In contrast, probabilities of Cry protein detection and percent growth inhibition were significantly higher in the cover crop, flail mow and undisturbed treatments, though differences among these treatments were not statistically significant.

The unexpected slower rate of degradation observed in the chisel plow treatment in 2014/2015 was most likely due to differences in the litter bags used each study year, as well as differences in how tightly the corn residue was packed into the bags. The mesh bags used in 2014/2015 had much smaller openings and were packed tighter than the onion bags used in 2016/2017 (Figure 13). Together, these factors may have impeded the ability of soil organisms to enter the litterbags during the first study year, resulting in decreased soil-tissue contact, litter decomposition and protein degradation. The loosely packed bags of the second year simulated more closely the soil incorporation of corn residue resulting from post-harvest tillage practices. Other factors could have contributed to differences observed in the chisel plow treatments between experiments, such as soil

composition, pH, and organic matter. For example, studies suggest that soils high in clays are more likely to bind Cry proteins and thus interfere with microbial degradation processes (Stotzky 2005). However, these factors unlikely contributed to the different effects between years because both experiments were conducted in the same field. Despite the inconsistencies in treatment effects, results of the second year supported the hypothesis that greater soil-residue contact in the chisel plow treatment resulted in most rapid degradation of Cry proteins.

Increased degradation was also predicted to occur in the cover crop treatment compared to the flail mow and undisturbed treatments due to increased residue-soil contact resulting from the incorporation of corn residue into the soil by the drill at planting. However, no significant differences in percent growth inhibition or mean weight gain of larvae exposed to Bt residue were observed in either year. The no-till drilling action of planter incorporated roughly 11% of the corn residue into the soil. However, because the sampling method collected only surface residue within a pre-measured area and not the soil-incorporated tissue in the cover crop treatment, the tissue used for bioassay and ELISA testing was virtually the same as that used in the flail mow treatment. Additionally, previous studies have shown that Cry proteins degrade more slowly under cooler temperatures (Bai et al. 2007, Feng et al. 2011, Li et al. 2007, Zhang et al. 2015). The presence of a cover crop canopy blocks sunlight and results in cooler surface temperatures (Montague and Kjelgren 2004), which could have negated any accelerated degradation caused by increased contact with the soil surface. Future

studies are needed to determine how cover crop planting impacts degradation rates of Cry proteins in post-harvest corn residue.

I also hypothesized that slower degradation of the Cry proteins would occur during winter months (weeks 12-22 after harvest), when temperatures are colder and soil microbes are less active. Ambient temperatures were different during this time between experiment years. Table 2 shows the average monthly temperature throughout each year, as well as the number of days per month when the daily temperature remained below 4°C, the generally accepted temperature below which microbial degradation ceases to occur (Rabenhorst 2005). In 2014/2015, there was a total of 42 days between late December (week 12) and early March (week 22) when daily temperatures remained below 4°C. Figures 4A and 5A show a relatively slower rate of change in mean larval weight gain and percent growth inhibition across all treatments during sampling weeks 12 through 22, except for the chisel plow treatment which showed continued degradation during this period. The litter bag tissue was likely exposed to soil temperatures that were less prone to fluctuations in surface temperatures; thus, the soil probably remained warmer after surface temperature dropped, allowing microbial activity and protein degradation to occur. For the second year, percent growth inhibition showed a steady decrease over the three winter months, during which only 17 days remained below 4°C (Figures 9A, 10A). This was probably not enough cumulative cold days to slow the rate of protein degradation. Relative changes in larval growth inhibition during the winter months of each year also provide evidence that colder temperatures in the first year slowed the rate of protein

degradation. For example, percent growth inhibition of larvae fed diet incorporated with the surface residue of the flail mowed plots declined during the winter months by 18.3% in the first year compared to 67.4% in the second year. These results suggest that temperature may have a greater influence on the rate of Cry protein degradation in no-till corn production where the majority of crop residue remains on the soil surface.

The growth inhibition results could not separate the relative activity of the two lepidopteran-active proteins against *O. nubilalis*, nor provide any information about coleopteran active proteins. However, ELISA results revealed consistent differences in the detection probabilities of the four Cry proteins. Of the two rootworm-active proteins, Cry3Bb1 degraded much faster than Cry34Ab1, which is coupled with Cry35Ab1. Although targeted for rootworms, these proteins are also expressed in the leaf tissue at equal to or higher concentrations than in root tissue. Depending on the corn growth stage, the titer of Cry3Bb1 and Cry34Ab1 ranges from 189-240 ug/g dwt and 50-220 ug/g dwt, respectively. However, the Cry3Bb1 concentration in the post-harvest residue or stover is considerably lower, ranging from 20-35 ug/g dwt (EPA 2010c, EPA2010d); so there is less protein present after harvest compared to the Cry34Ab1 protein. Moreover, Cry34Ab1 may take longer to degrade because it is coupled with Cry35Ab1; but, it is unclear whether a negative ELISA test for Cry34Ab1 also means that Cry35Ab1 is not present. In any case, the persistence of coleopteran active proteins in aboveground tissue was not expected and suggests that both soil and epigeal organisms could be exposed to these active proteins for a prolonged period after harvest. Because

the roots of SmartStax corn remain intact in a no-till production system, further research is needed to quantify the release and persistence of the coleopteran active proteins from root tissue after harvest. The Cry2A protein showed an overall lower probability of detection during the early sampling weeks compared to the Cry1F protein, which suggests that the observed larval growth inhibition during the later weeks could be largely due to Cry1F.

I predicted that ELISA tests would indicate positive detections of the lepidopteran active proteins beyond the point of any evidence of larval growth inhibition. Previous research found that ELISA tests can produce false positives as a result of biologically inactive Cry protein fragments (Marchetti et al. 2007, Gruber et al. 2011). However, this did not occur because there was significant larval growth inhibition during the later sampling weeks after ELISA stopped detecting the presence of the Cry1F and Cry2A proteins. In 2014/2015, 70% growth inhibition was observed in larvae reared on Bt tissue collected from the undisturbed treatment at 22 weeks after harvest; yet, ELISA tests failed to detect the presence of the proteins in any treatment on the same week. *O. nubilalis* larvae are exceptionally sensitive to Cry proteins (Huang et al. 2006) so the low concentrations used in the bioassay experiments were sufficient to detect biological activity. However, growth inhibition may have been even more pronounced in later weeks if greater concentrations of Bt tissue were used. Consequently, it is likely that the remaining levels of biological activity of the Cry proteins may have been underestimated as a result of the low concentrations of residue tissue used in the bioassays.

Some general comments can be made about the sampling protocol that could have affected the results. First, protein degradation among post-harvest treatments, as indicated by *O. nubilalis* growth inhibition and protein detection, could have been influenced by differences in sampling methods between the experiment years. In the first year, leaf residue was collected in all plots, except for samples taken from the flail mowed treatment which also contained husk, cob and stalk tissue. Cry protein expression in Bt corn is generally higher in leaf tissue compared to the roots and other aboveground plant parts (Clark et al. 2005). Therefore, incorporating tissue from all parts of the plant may have diluted the titer levels of detectable Cry proteins, resulting in lower percent inhibition and probability of detection in the flail mow treatment. Secondly, as previously mentioned, because samples of plant residue from the cover crop plots did not include the portion of tissue incorporated in the soil, percent growth inhibition could have been slightly overestimated. Thirdly, the litter bag method differed between years and apparently had a major impact on protein degradation in the chisel plow treatment. Although litter bags provided a convenient tool to collect residue over the sampling period, future studies should consider an alternative method that mimics more closely the distribution and soil contact of residue when incorporated by moldboard or deep chisel plow practices. Lastly, the bioassays were conducted at the same time on all replicate samples collected from all treatments at a given week or groups of weeks. Although the same protocol was followed, there may have been variations in the bioassay process, particularly the initial weight of the test larvae that could have affected the weight gain. Although

relative growth inhibition was less affected, the unusual changes in the pattern of larval weight gain shown in Figure 8A most likely reflects differences in the initial weight of larvae between groups of bioassay runs. In hindsight, processing all treatment samples of one replicate over the entire sample one of the time would be better approach in order to statistically adjust for experimental error among bioassay runs.

In summary, results from this study found that Cry proteins retain biological activity much longer after harvest in no-till corn systems than was previously thought. Although few negative effects on non-target organisms have been documented to date, the potential for nontarget effects from future genetically engineered varieties exists, particularly in no till corn systems that leave crop residue on the soil surface. Postharvest practices that promote increased soil-residue contact, and therefore faster protein degradation may help to mitigate these effects by reducing the period of exposure for soil organisms.

# Tables

| Growth Stage | % Inhibition | Larval Weight (Bt) | Larval Weight (Non-Bt) |
|---|---|---|---|
| R1 | 98.82% | 0.597 µg | 31.391 µg |
| R6 | 97.96% | 0.608 µg | 50.568 µg |

**Table 1.** Growth inhibition and larval weights of 2nd instar *O. nubilalis* after feeding for 7 days on diet incorporated with lyophilized Bt and non-Bt corn tissue collected at the R1 and R6 growth stages in 2014-2015.

| | 2014/2015 | | 2016/2017 | |
|---|---|---|---|---|
| **Sample Month** | **Average Temp °C** | **Days < 4°C** | **Average Temp °C** | **Days < 4°C** |
| **September** | 19.9 | 0 | 21.6 | 0 |
| **October** | 14.2 | 0 | 14.9 | 0 |
| **November** | 6.2 | 4 | 8.8 | 0 |
| **December** | 4.2 | 4 | 3.2 | 7 |
| **January** | -0.7 | 15 | 4.0 | 6 |
| **February** | -3.4 | 16 | 7.3 | 4 |
| **March** | 4.7 | 3 | 6.8 | 0 |

**Table 2.** Average monthly temperature (°C) throughout the sampling period during 2014/2015 and 2016/2017 as well as the number of days per month in each study year where the daily temperature did not rise above 4°C. Weather data collected from a weather station located at the Central Maryland Research and Education Center, Beltsville Facility.

Figures

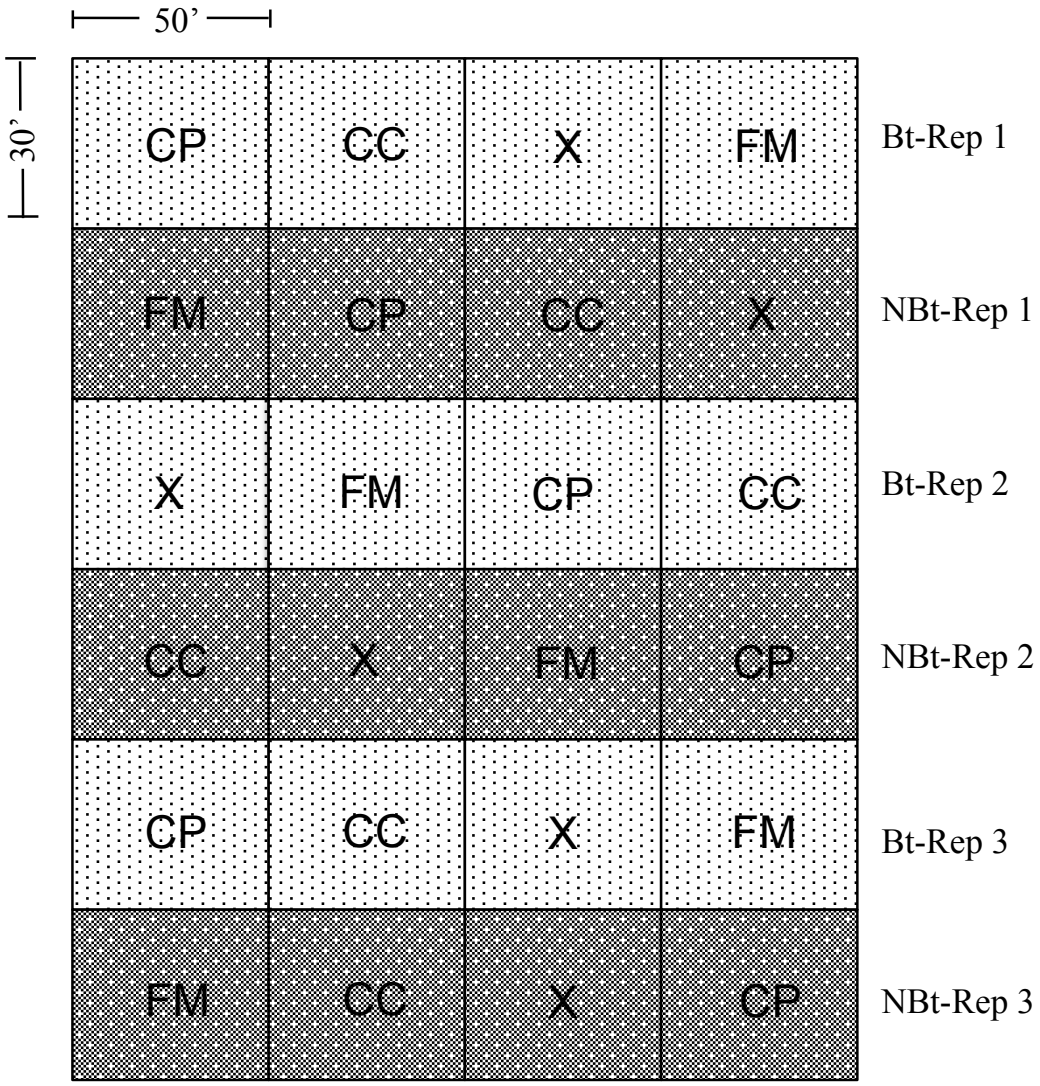


**Figure 1**. Field layout of four post-harvest treatments replicated three times at Beltsville in 2014-2015. Each replicate contained 12 rows of Bt SmartStax field corn and 12 rows of the nearest non-Bt isoline. CP = chisel plow, CC = cover crop, X = undisturbed and FM = flail mow.

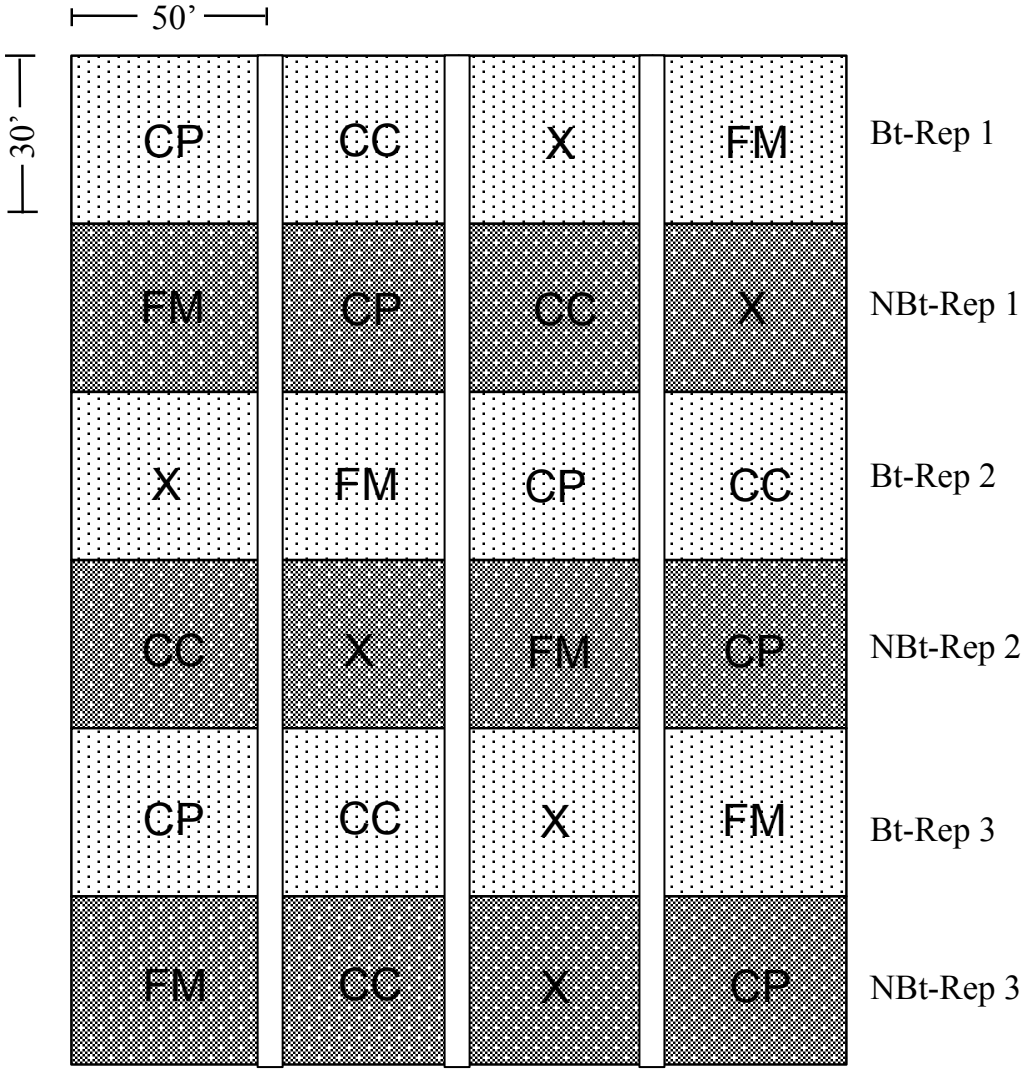


**Figure 2**. Field layout of four post-harvest treatments replicated three times at Beltsville in 2016-2017. Each replicate contained 12 rows of Bt SmartStax field corn and 12 rows of the nearest non-Bt isoline. Treatments were separated by a 30 foot alleyway. CP = chisel plow, CC = cover crop, X = undisturbed and FM = flail mow. (Figure not to scale).

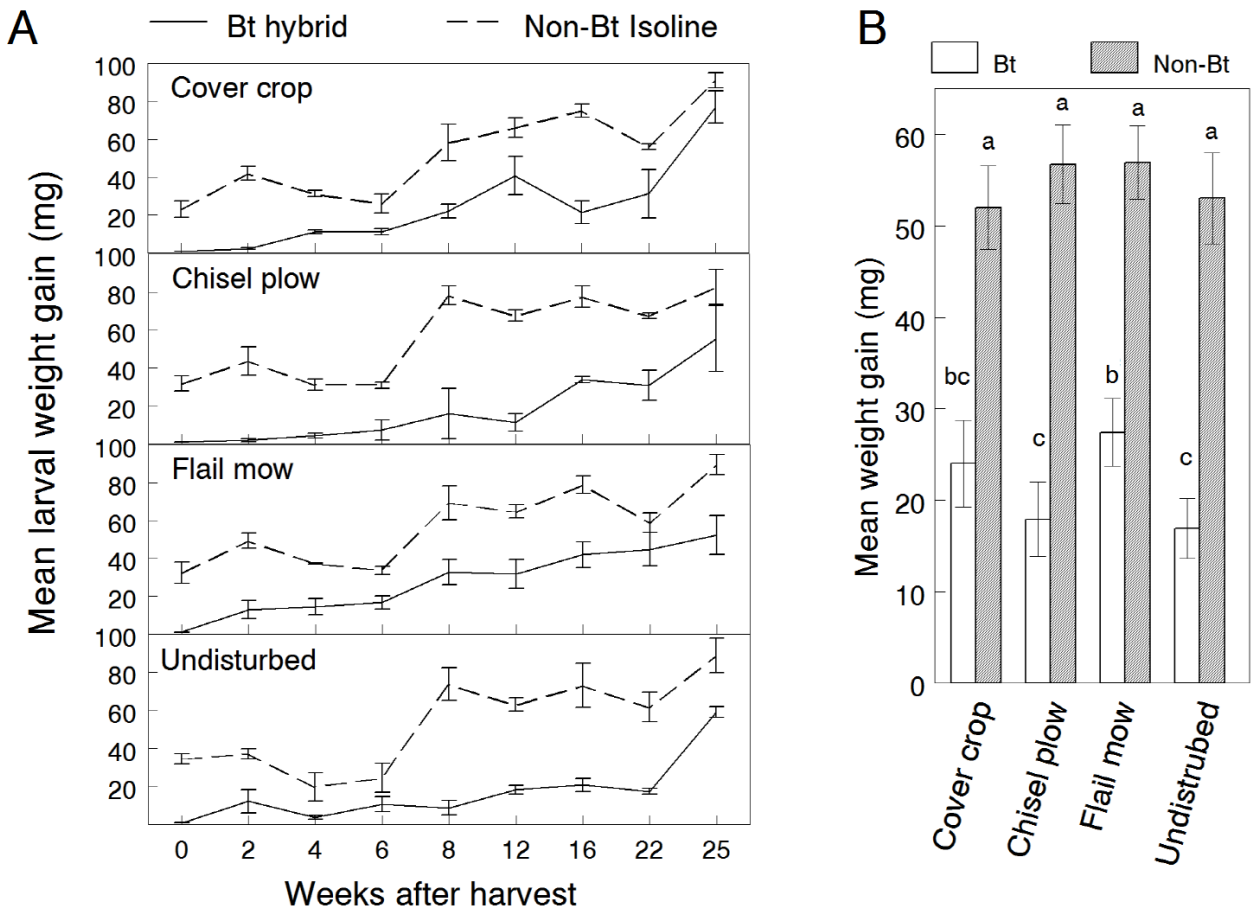


**Figure 3**. Mean body weight gains (±SEM) of early 2nd instar *O. nubilalis* after feeding for 7 days on diet incorporated with lyophilized Bt and non-Bt corn residue collected over 25 weeks post-harvest from plots managed under different postharvest treatments. Graph A shows the hybrid by treatment by week means, whereas the graph B shows the hybrid by treatment means pooled across sampling weeks. Mean bars bearing the same letter are not significantly different at the 5% probability level. Beltsville 2014-2015.

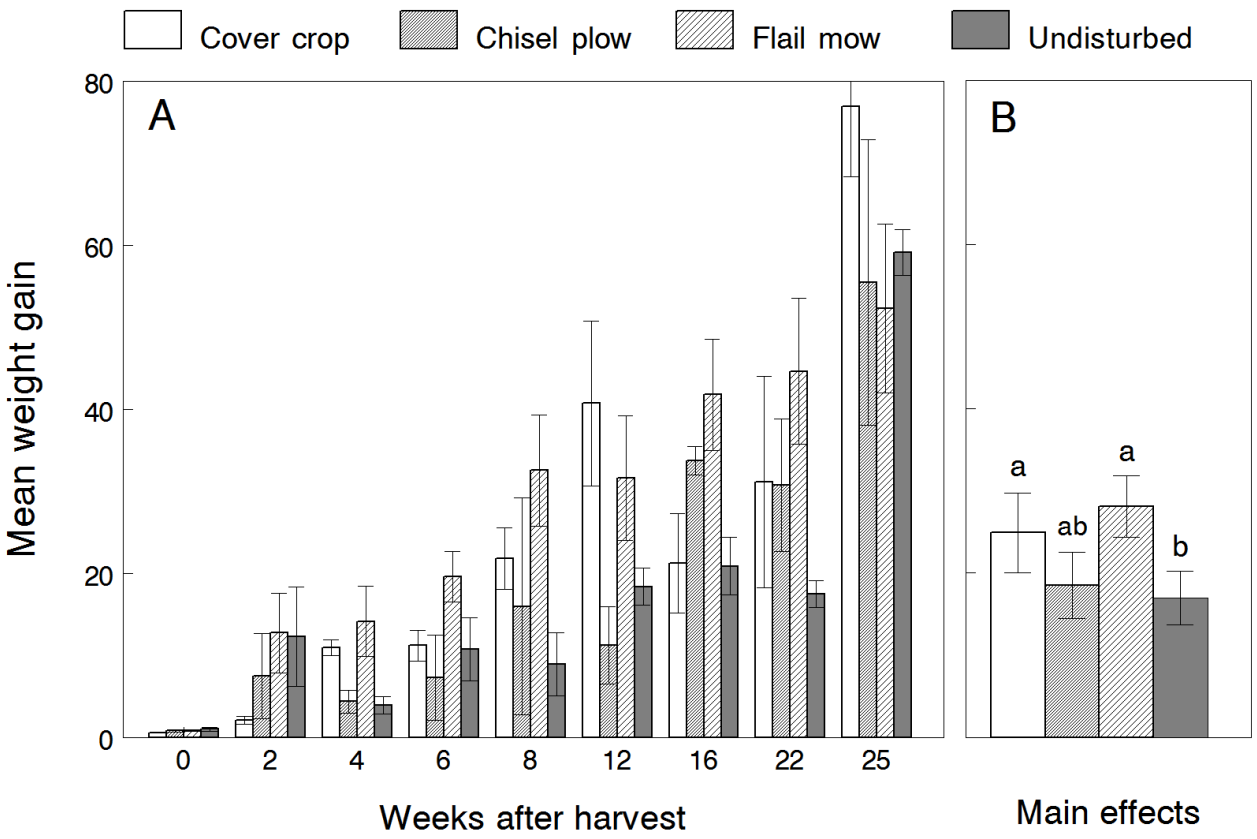


**Figure 4.** Mean body weight gains (±SEM) of early 2nd instar *O. nubilalis* after feeding for 7 days on diet incorporated with lyophilized Bt residue collected over 25 weeks post-harvest from plots managed under different postharvest treatments. Graph A shows the hybrid by treatment by week means, whereas the graph B shows the main effect treatment means pooled across sampling weeks. Mean bars bearing the same letter are not significantly different at the 5% probability level. Beltsville 2014-2015.

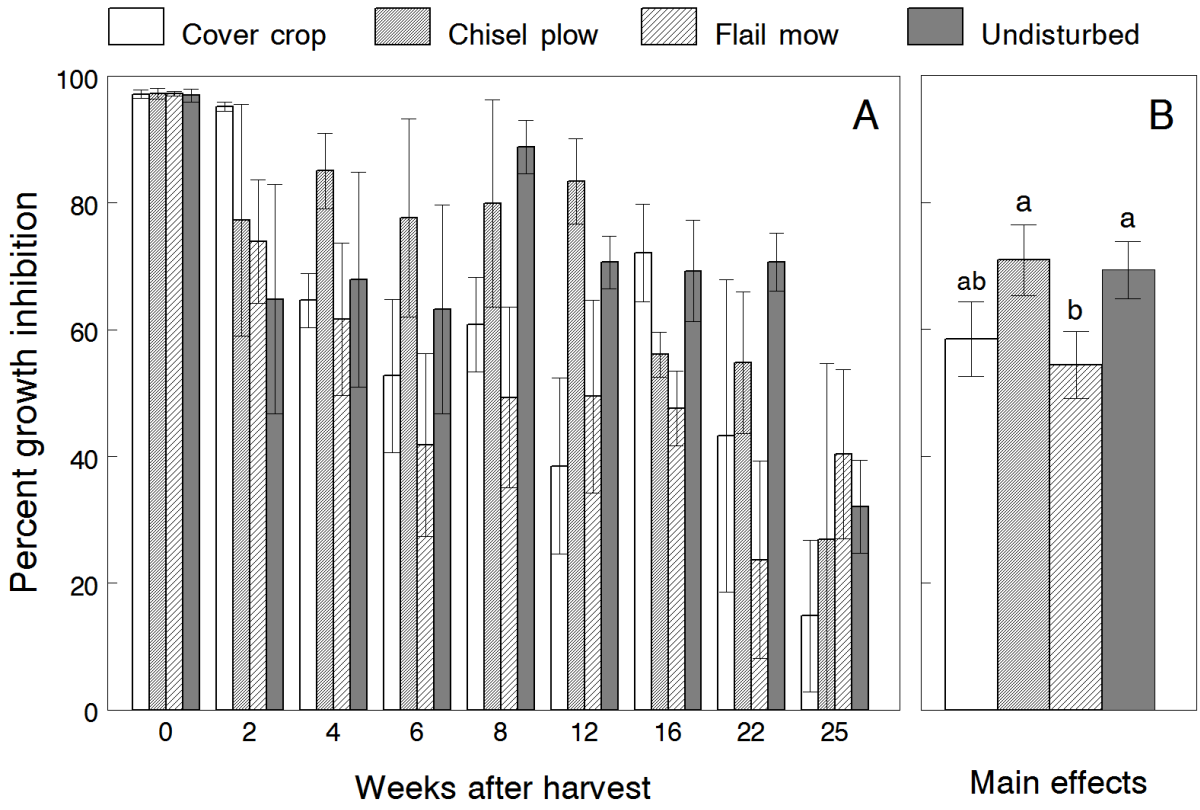


**Figure 5.** Mean (±SEM) percent growth inhibition of early 2nd instar *O. nubilalis* after feeding for 7 days on diet incorporated with lyophilized Bt residue collected over 25 weeks post-harvest from plots managed under different postharvest treatments. Percent inhibition was calculated as the difference in weight gain of larvae feeding on diets incorporated with Bt corn residue relative to those larvae exposed to non-Bt residue. Graph A shows the hybrid by treatment by week means, whereas the graph B shows the main effect treatment means pooled across sampling weeks. Mean bars bearing the same letter are not significantly different at the 5% probability level. Beltsville 2014-2015.

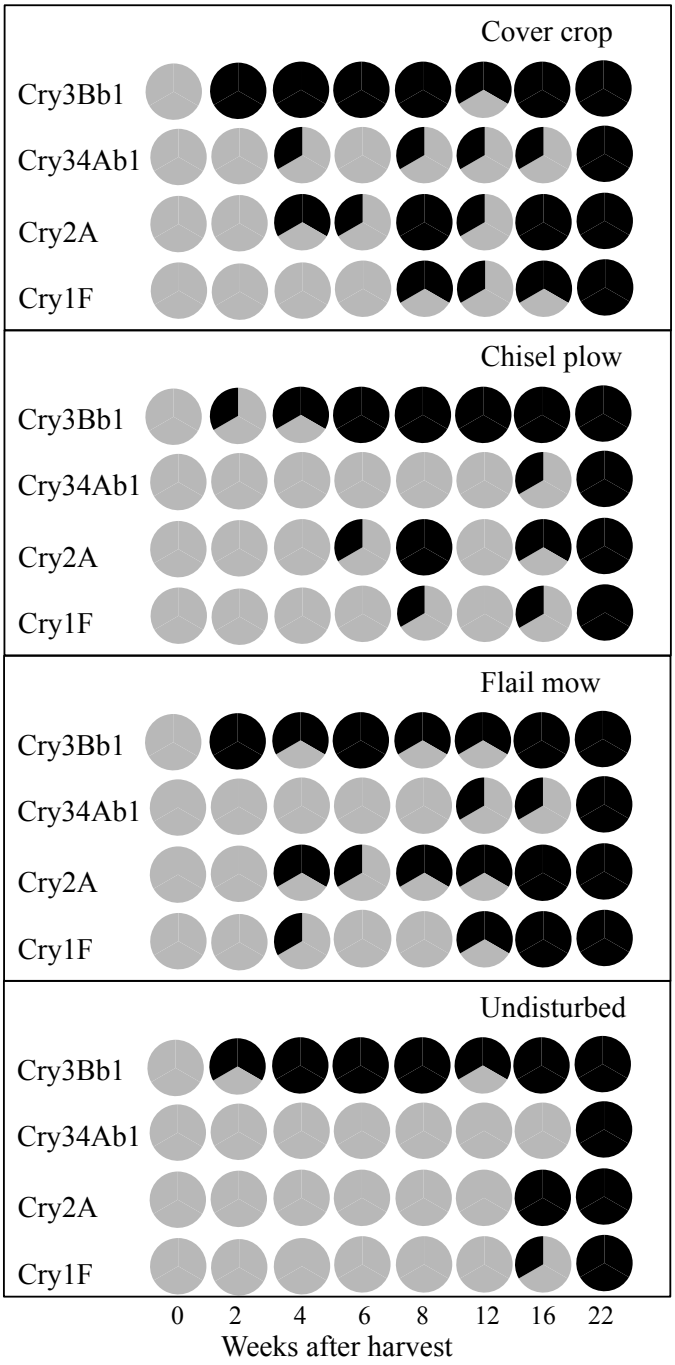


**Figure 6.** Raw data from individual tests to detect the presence of Cry3Bb1, Cry34Ab1, Cry2A and Cry1F proteins in SmartStax field corn using an enzyme-linked immunosorbent assay (ELISA). Three replicate samples were tested for each combination of postharvest treatment and sampling week after harvest. Gray pie sections indicate positive detections, while black sections indicate negative detections. Beltsville 2014-2015.

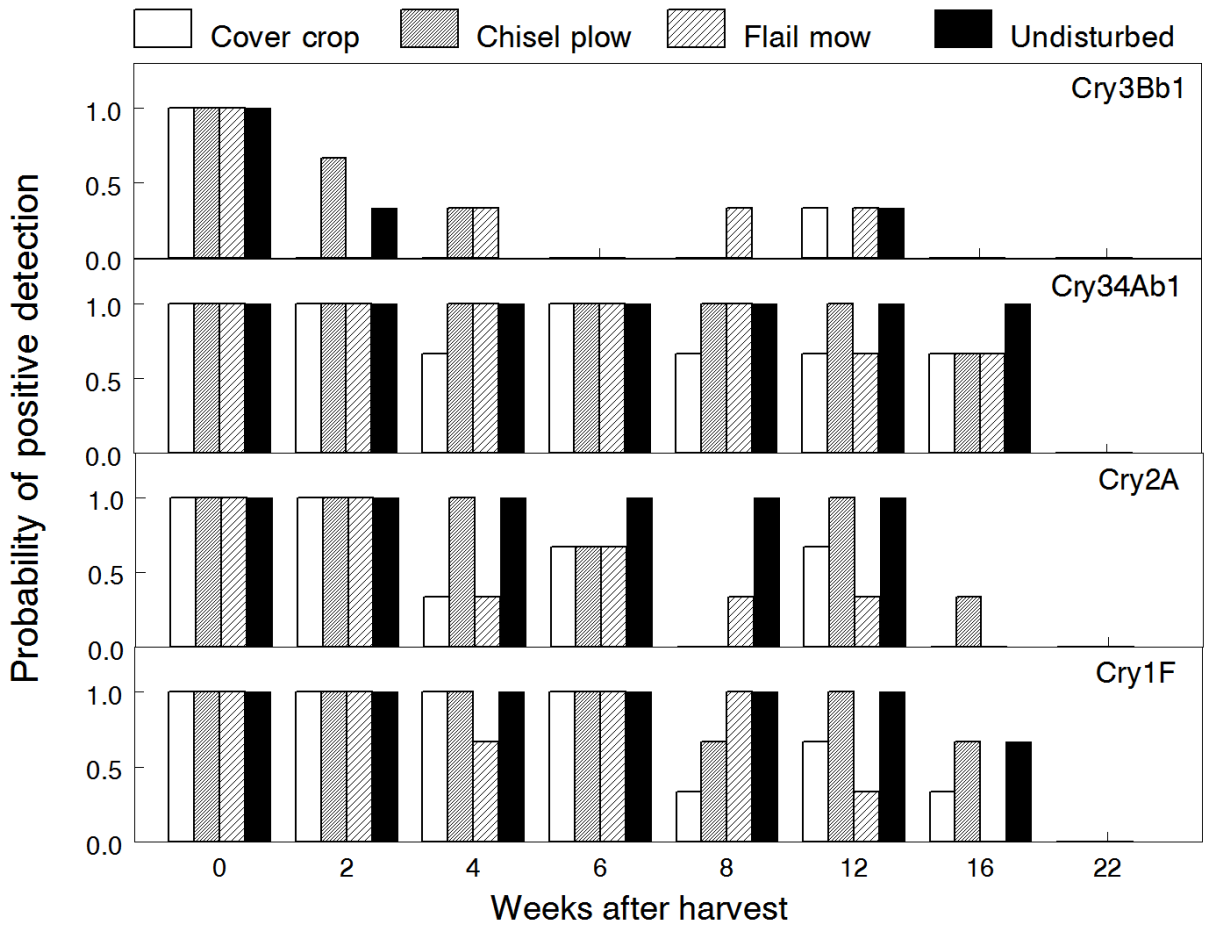


**Figure 7.** Probability of positive detection of Cry3Bb1, Cry34Ab1, Cry2A and Cry1F proteins in SmartStax field corn based on a quantitative enzyme-linked immunosorbent assay (ELISA). Means of each protein are given for each postharvest by sampling week combination. Beltsville 2014-2015.

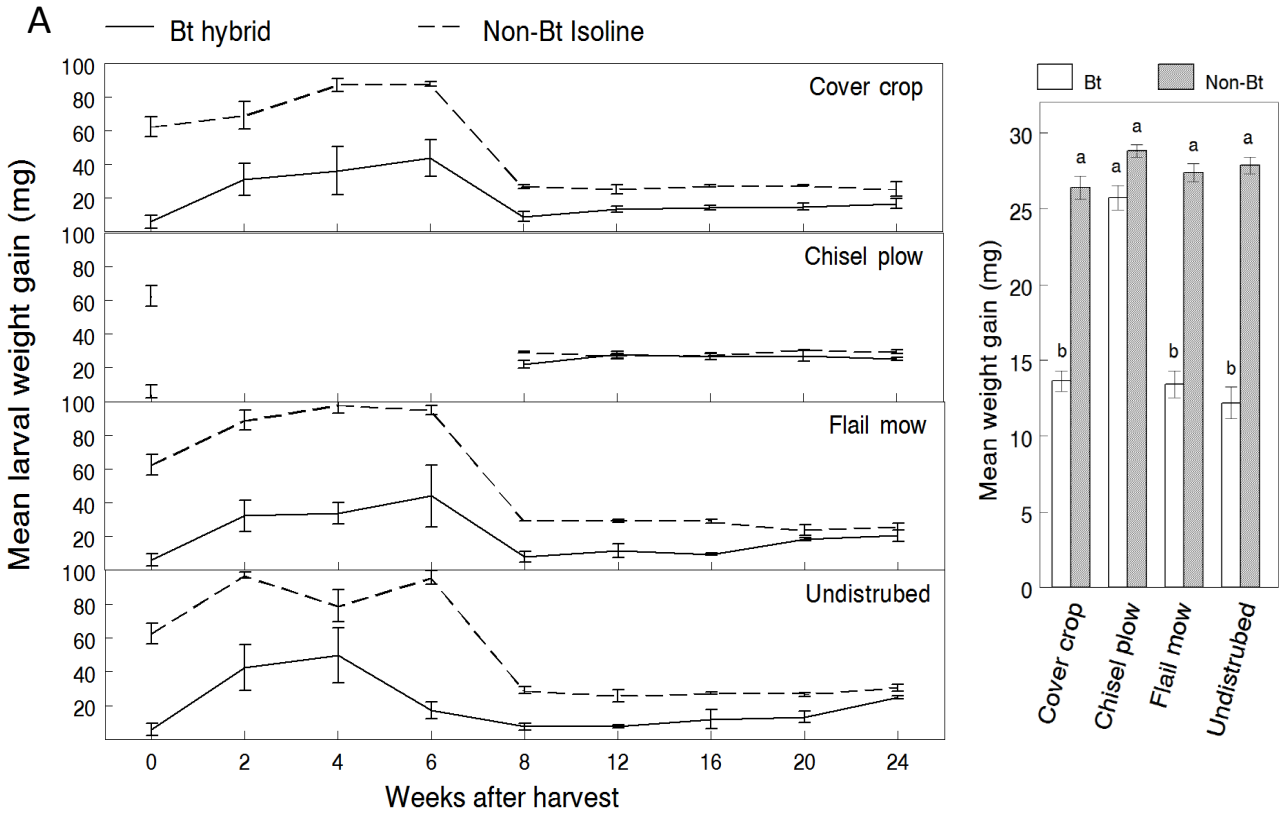


**Figure 8.** Mean body weight gains (±SEM) of early 2nd instar *O. nubilalis* after feeding for 7 days on diet incorporated with lyophilized Bt and non-Bt corn residue collected over 24 weeks post-harvest from plots managed under different postharvest treatments. Graph A shows the hybrid by treatment by week means, whereas Graph B shows the hybrid by treatment means pooled across sampling weeks. Mean bars showing the same letter are not significantly different at the 5% probability level. Beltsville 2016-2017.

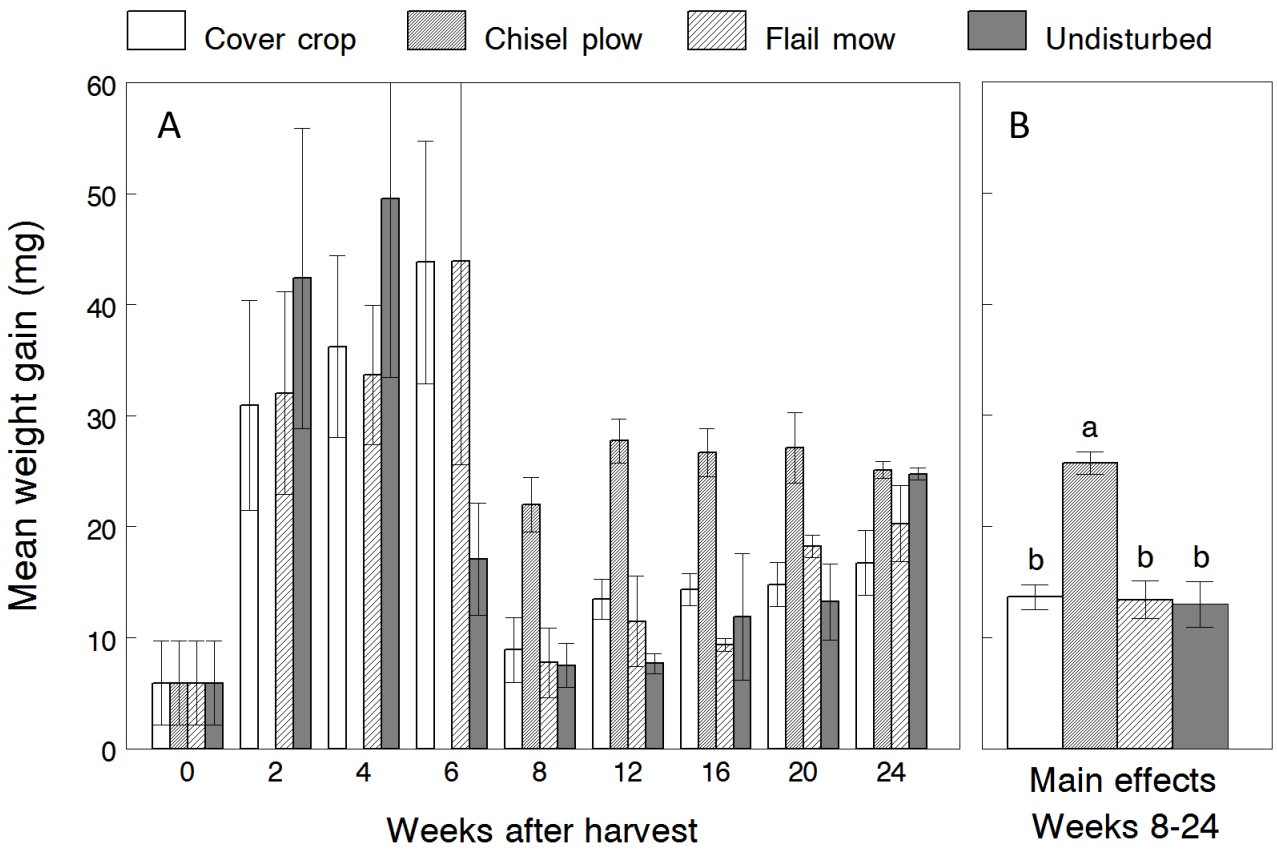


**Figure 9.** Mean body weight gains (±SEM) of early 2nd instar *O. nubilalis* after feeding for 7 days on diet incorporated with lyophilized Bt residue conducted over 24 weeks post-harvest from plots managed under different postharvest treatments. Graph A shows the hybrid by treatment by week means. Graph B shows the main effect treatment means pooled across sampling weeks. Mean bars bearing the same letter are not significantly different at the 5% probability level. Beltsville 2016-2017.

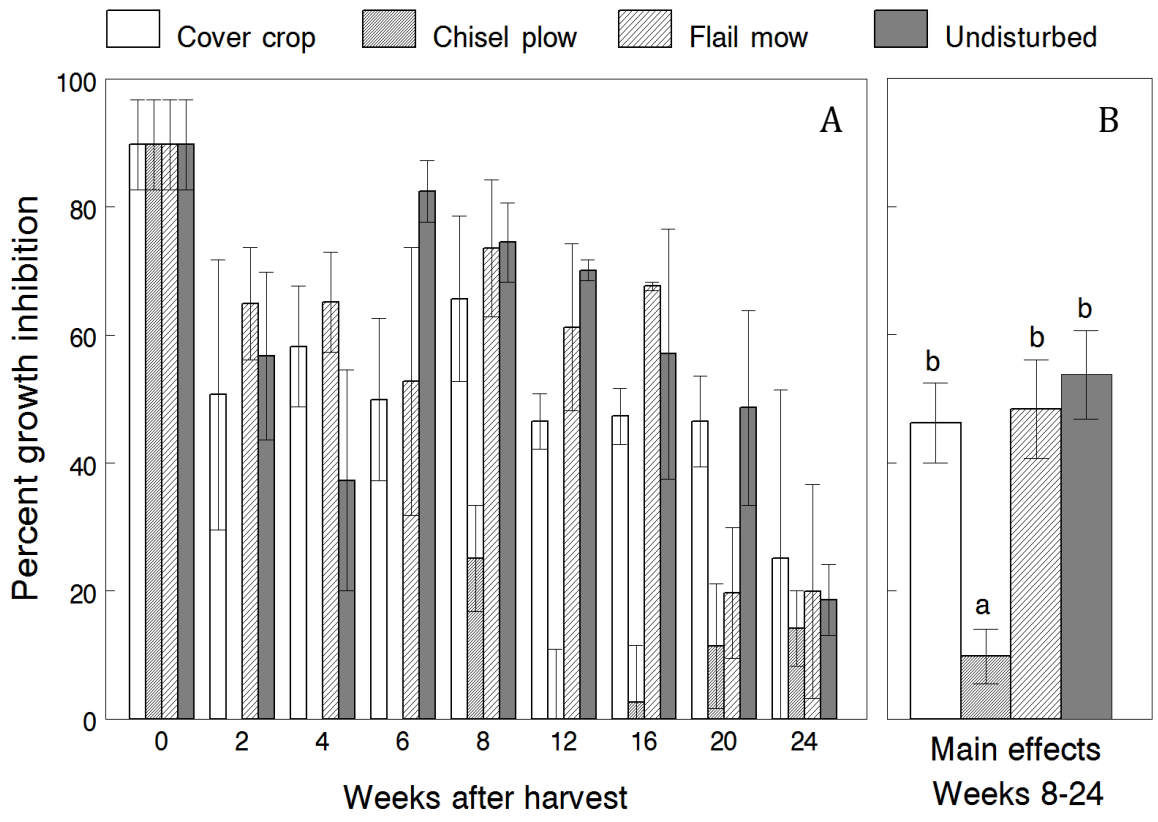


**Figure 10.** Mean (±SEM) percent growth inhibition of early 2nd instar *O. nubilalis* after feeding for 7 days on diet incorporated with lyophilized Bt residue collected over 24 weeks post-harvest from plots managed under different postharvest treatments. Percent inhibition was calculated as the difference in weight gain of larvae feeding on diets incorporated with Bt corn residue relative to those larvae exposed to non-Bt residue. Graph A shows the hybrid by treatment by week means, whereas the Graph B shows the main effect treatment means pooled across sampling weeks. Mean bars bearing the same letter are not significantly different at the 5% probability level. Beltsville 2016-2017.

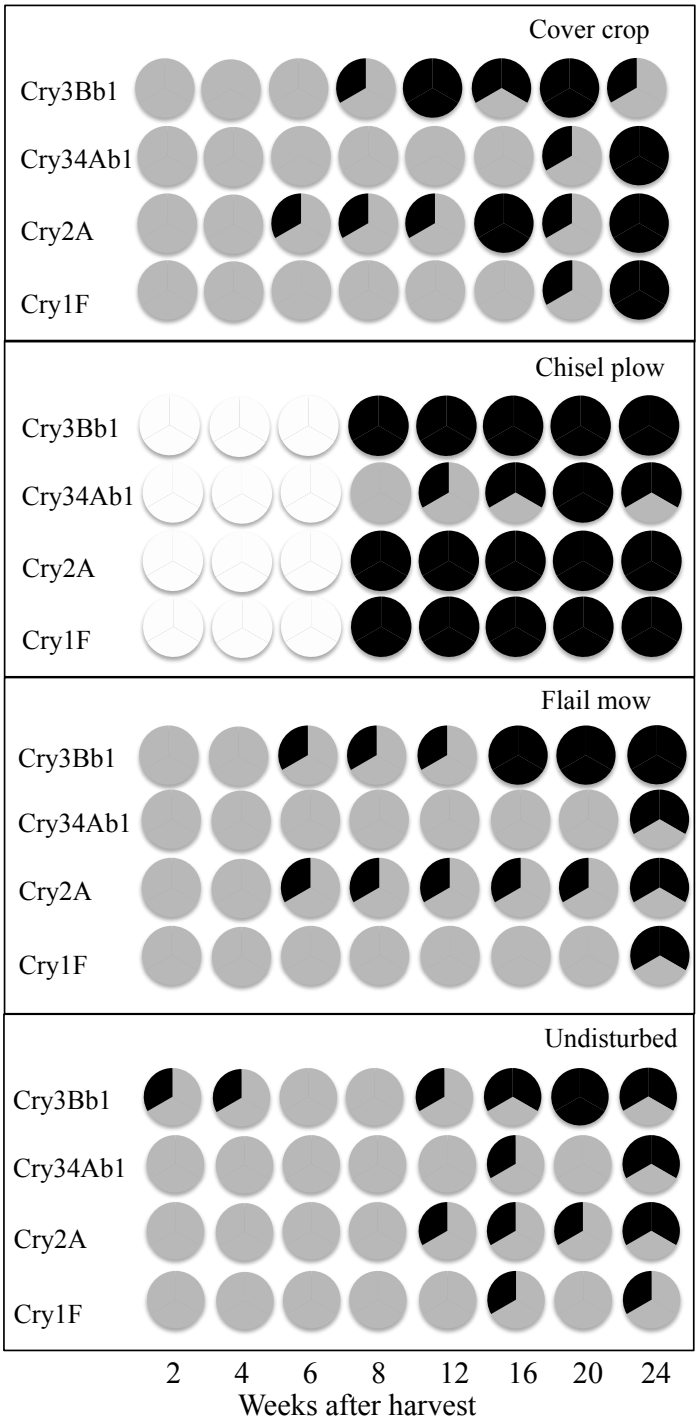


**Figure 11.** Raw data from individual qualitative ELISA tests indicating the presence of Cry3Bb1, Cry34Ab1, Cry2A and Cry1F proteins in SmartStax field corn residues. Three replicate samples were tested for each combination of postharvest treatment and sampling week after harvest. Gray pie sections indicate positive detections, while black sections indicate negative detections. Beltsville 2016-2017.

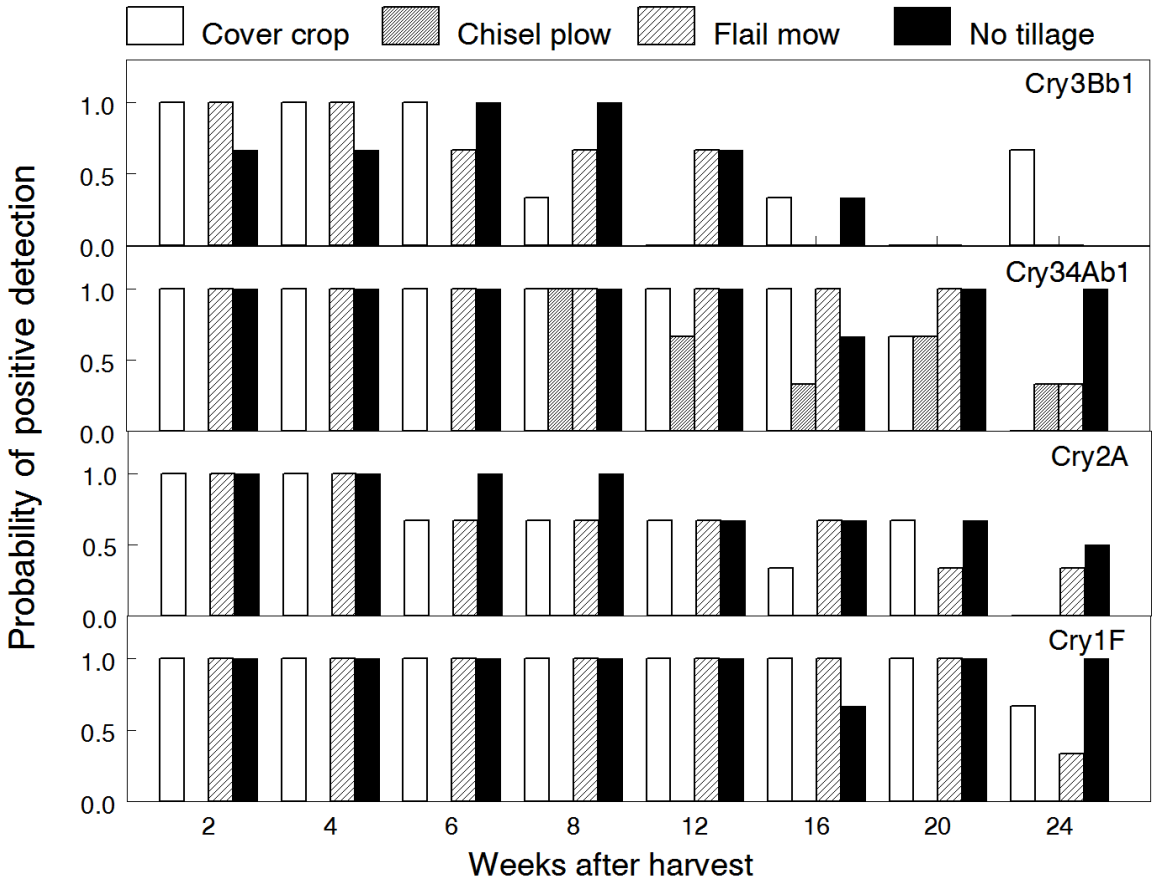


**Figure 12.** Probability of positive detection of Cry3Bb1, Cry34Ab1, Cry2A and Cry1F proteins in SmartStax field corn based on quantitative enzyme-linked immunosorbent assay (ELISA). Means of each protein are given for each postharvest treatment by sampling week combination. Beltsville 2016-2017.


**Figure 13.** Image A shows the litter bags used in the chisel plow treatment during 2014-2015 while image B shows the larger, more open mesh litterbags used during 2016-2017.

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